Demonstration of de novo production of adipocytes in adult rats by biochemical and radioautographic techniques

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Abstract Adult rats of various strains show small increases in the number of adipocytes in the epididymal (Epi) fat pad and large increases in the retroperitoneal (RP) fat depot when they are fed a high-fat, high-sugar diet (HFS). In the present study, radioautographic techniques are used to demonstrate that these increases result from replication and differentiation of adipocyte precursors. In addition, both in vivo and in vitro biochemical techniques are used to demonstrate that HFS-induced acceleration of DNA synthesis differs between Epi and RP depots. Measures of levels of incorporation of radiolabeled thymidine into DNA in rats fed HFS for 4 weeks reveal significant elevation in the RP depot but not in the Epi pad. Analysis of radioautographs indicates that formation of precursors may be accelerated in the RP depot as early as the first week of HFS-feeding. Results obtained from the in vitro biochemical assay are of interest with regard to possible clinical application since current in vivo techniques for quantitative assessment of precursor synthesis cannot be applied directly to the study of adipose tissue growth in man. While data obtained from the in vitro assay contains a relatively high degree of variation, the information it provides is in general agreement with that provided by the in vivo assay.—**Miller, W. H., Jr., I. M. Faust, and J. Hirsch.** Demonstration of de novo production of adipocytes in adult rats by biochemical and radioautographic techniques. *J. Lipid Res.* **25:** 336–347.

Supplementary key words obesity • adipose tissue • DNA synthesis

Recent experiments have established that large increases in the number of adipocytes occur in adult, genetically obese rats and can be induced in adult rats of common laboratory strains by the feeding of diets with a high fat or high sugar content (1, 2). There also appear to be circumstances in which the number of adipocytes increases during adulthood in humans (3, 4). It has been suggested that such increases may be the result of maturation and lipid-filling of fat cells recruited from a pool of pre- (or very small) adipocytes formed early in life (5). The other possibility is that an increasing number of fat cells results from the re-initiation or acceleration of cell multiplication and differentiation. Several lines of evidence favor the latter view.

Cleary et al. (6, 7) found that the activity of thymidine kinase, an enzyme associated with DNA synthesis, is elevated in the epididymal (Epi) pad of genetically obese Zucker rats during adulthood. Klyde and Hirsch (8, 9) found significant increases in incorporation of [^{3}H]thymidine in both stromal-vascular (SV) and adipocyte fractions of Epi and retroperitoneal (RP) fat depots of adult Osborne Mendel (OM) rats fed HFS. The present set of studies is aimed at demonstrating conclusively whether or not de novo production of fat cells occurs in adult rats as well as whether biochemical measures of DNA synthesis in adipose tissue can be used to assess the rate of that production.

Epi and RP depots differ markedly in the degree to which they show increases in adipocyte number in rats fed fattening diets. The Epi pad usually shows a significant but late and limited increase in fat cell number, while the RP depot consistently shows an increase that appears earlier than in other depots and continues at a high rate for many months (2, 10). If increased levels of DNA synthesis in adipose tissue reflect the production of new adipocytes, Epi and RP depots should show different levels of DNA synthesis as well. The first study was designed to determine whether there is such a difference between these two depots. Of course, an observation of differences in DNA synthesis between RP and Epi depots is not sufficient to demonstrate de novo adipocyte production conclusively. Differential increases in DNA synthesis might also be seen if there were different degrees of proliferation of SV cells required for the support of fat cells newly recruited from a pool of pre-adipocytes. Therefore, in the second study, we used the method of radioautography to distinguish synthesis of adipocytes from synthesis of SV cells.

The results of the first two studies indicate that increased levels of DNA synthesis in adipose tissue of adult

Abbreviations: Ara-C, cytosine arabinoside; Epi, epididymal; HFS, high-fat, high-sugar diet; OM, Osborne Mendel; RP, retroperitoneal; SV, stromal-vascular; KRB, Krebs Ringer bicarbonate buffer.

rats fed a fattening diet do indeed reflect production of new adipocytes. Measures of DNA synthesis in adipose tissue might also prove to be useful for the assessment of adipocyte production in humans; but techniques used to study in vivo cell production in the rat cannot be applied to humans, since they require the injection of large doses of radioactive DNA precursor. However, there is evidence that DNA synthesis can be measured in adipose tissue pieces incubated in vitro (8, 11) and subcutaneous adipose tissue, which is abundant in humans, can be sampled safely and simply by needle aspiration. Therefore, in the third study, we examined whether an in vitro assay would provide information consistent with that found with the more direct in vivo measures.

METHODS

Depot-specific DNA synthesis in vivo

Thirty male OM rats were obtained as weanlings from Rockland Farms (Gilbertsville, PA). They were housed at the Rockefeller University Laboratory Animal Research Center under standard conditions and fed Purina laboratory chow ad libitum. At 3 months of age, 18 of the rats were used to study the short-term effects of a fattening diet. They were divided into three weight-matched groups of six rats per group. One group (control) was continued on chow while the other two groups (experimental) were switched to a high-fat, high-sugar diet (HFS) consisting of chow, Borden's sweetened condensed milk (diluted 1:1 with water), Chips Ahoy chocolate chip cookies (Nabisco), and water. Purina chow is a complete rat diet containing (by weight) approximately 25% protein and 3% fat. It provides about 3.5 calories per gram. Undiluted Borden's milk contains (by weight) approximately 7% protein and 9% fat and provides about 3 calories per gram. The chocolate chip cookies contain (by weight) approximately 5% protein and 40% fat and provide about 5 calories per gram. One experimental group was fed the fattening diet for 5 days, and the other for 10 days. The remaining 12 rats of the original 30 were used to study the long-term effects of the fattening diet. They were divided into two weight-matched groups at 4 months of age. One group (experimental) was fed the above HFS diet ad libitum for 5 weeks, while the other (control) remained on chow.

After the rats of the 5- and 10-day HFS groups had been on the diet for 3 and 7 days, respectively, they and their controls were each injected intraperitoneally with 250 μCi of [^{3}H]methyl thymidine (New England Nuclear, Boston, MA). This dose does not affect food intake, but does yield high counts above background in extracted tissue. After 4 weeks on HFS, experimental and control rats in the long-term study were each injected with a total of 600 μCi of [^{3}H]thymidine in 200-μCi doses administered daily for 3 days. Since this was a long-term experiment, it was possible to use multiple injections to decrease the variance in counts which can result from variation in the uptake of label. All rats were killed at the end of their respective experimental periods, and Epi and RP fat depots were removed by dissection, weighed, and sampled for lipid content and cellularity determinations as previously described (12), and for determinations of DNA synthesis as follows. Samples (100–200 mg) of tissue were extracted three times with double-distilled diethyl ether to remove all lipid. The lipid-free tissue was first extracted with 10% trichloroacetic acid and then with 95% ethanol to remove all non-incorporated labeled precursors. The tissue was then dissolved in NCS protein solubilizer (Amersham, Arlington Heights, IL) and counted in a scintillation counter. Incorporation of label into DNA, as measured by this technique, was found to be highly correlated ($r = 0.92$) with DNA specific activity measured with much larger samples of tissue processed by the more elaborate extraction and purification procedures described by Klyde and Hirsch (8). The value of the present technique is that it can be used to assay very small pieces of adipose tissue such as are obtained by needle aspiration from humans (and incubated with [^{3}H]thymidine in vitro, see below). Incorporation of [^{3}H]thymidine is expressed in terms of dpm/mg tissue, dpm/fat pad, and dpm/fat cell.

Radioautographic confirmation of de novo production of adipocytes

OM male rats were obtained as weanlings from Rockland Farms and housed as described in the previous section. In the first experiment, nine 7-month-old rats were divided into three weight-matched groups (two experimental, one control) of three each. The control group was fed chow ad libitum throughout the experiment. One experimental group was given ad libitum access to the HFS diet for 38 days. During the first 19 days on the diet, rats received nine injections of 100 μCi of [^{3}H]thymidine on alternate days. Rats in the second experimental group were given the HFS diet for 14 weeks and 12 injections of 100 μCi of [^{3}H]thymidine each, spaced evenly over days 19–46 of the HFS-feeding period. One rat in this group and one in the control group lost weight during the injection period and therefore were dropped from the experiment. Rats were killed at least 2 weeks after the final injection of radiolabel. RP adipose depots were removed by dissection and sampled for determination of cellularity and DNA synthesis as described above. In addition, samples of adipose tissue were fixed in 10% buffered formalin and embedded in JB4 embedding medium (Polysciences, Warrington, PA). Standard

embedding procedures did not lead to adequate infiltration of the resin into the adipose tissue, so they were modified as follows. Samples were dehydrated by successive 1-hr infiltrations with 70% ethanol and 95% ethanol followed by 3 × 30 min in 100% ethanol. Lipid was extracted by three 30-min washes of propylene oxide (Kodak). One hour of infiltration with a 1:1 mixture of propylene oxide and the glycol-methacrylate resin was followed by two infiltrations of 1 hr each with the resin. The tissue was then embedded, and 1-micron sections were cut on a JB4 microtome (Sorval), dipped into NTB-2 emulsion (Kodak), incubated for 5 months, and developed and stained with a solution of methylene blue and azure II. For each adipose depot examined, a minimum of 1000 nuclei from all technically adequate slides (at least five per depot) were examined for the presence of incorporated radiolabel and cell type. There was a relatively low level of label uptake into adipose tissue in these rats and virtually no background silver grains were observed. Therefore, nuclei showing three or more silver grains were highly distinct and were considered to be labeled (i.e., were considered as having [^{3}H]thymidine incorporated into DNA). All observed nuclei were classified as belonging to either fat cells, stromal-vascular (mesenchymal or endothelial) cells, or indeterminate cells as previously described (13). The number of indeterminate cells was never greater than 5% of those observed.

In a second experiment, nine 4-month-old male OM rats were divided into three groups of three each. Two groups were fed HFS and one group was fed chow. After one of the HFS groups had been fed the diet for 26 days, the second group of rats was started on the diet. From each experimental group, two rats that had consistently gained weight on the HFS diet and two controls were selected to receive two injections each of 500 μCi of [^{3}H]thymidine. An increased dose of radiolabel was found useful to shorten the time necessary for development of radioautographs. Rats in the first HFS group were injected after 3 and 5 days on the diet; rats in the second HFS group were injected after 29 and 31 days on the diet. All rats were continued on their respective diets for 3 weeks after the second injection. They were then killed and their Epi and RP adipose depots were dissected and sampled for DNA synthesis and radioautographic analysis as above, except that slides were developed after 1 month.

In vitro DNA synthesis

Adipose tissue was dissected from adult OM male rats that had been received as weanlings from Rockland Farms. This tissue was used in tests of an in vitro assay devised for the purpose of comparing levels of DNA synthesis in small adipose tissue fragments such as can be obtained by biopsy in humans. Beginning at ages 10–13 weeks, randomly selected groups of two or three rats (weight-matched to two controls) were fed HFS for periods of time ranging from 3 to 42 days. Controls were fed only chow and water. One or two control rats and one to three HFS-fed rats that had shown good weight gain on the diet were killed at various times after the initiation of HFS feeding. Rats that fail to gain weight on HFS also fail to show an increase in fat cell number (14), so measurement of levels of DNA synthesis in animals not gaining weight might have confounded the results. HFS-fed rats were selected for analysis only if they had gained at least 50% more weight while on HFS than chow-fed control rats. Epi and RP adipose depots were dissected and small (100–200 mg) pieces of tissue were incubated as follows. A tissue sample was minced with sharp scissors in physiological saline at 37°C, collected on a 250 μm mesh filter, and put into 4 ml of Krebs Ringer bicarbonate buffer (KRB) containing 3 μg/ml of chloramphenicol, 100 μg/ml of streptomycin, and from 0.1 to 1.0 μCi/ml of [^{3}H]methyl thymidine (New England Nuclear). This mixture was incubated for 60 min in a 37°C shaker bath. The adipose tissue fragments were collected and washed with physiological saline on a 250-μm mesh filter, reincubated with KRB plus 1.6 mM unlabeled thymidine for 20 min, washed, and incubated with unlabeled thymidine for 10 min more. The tissue was then collected and processed for isotope determination as in the in vivo DNA synthesis experiments above. In control experiments, adipose tissue was incubted with [^{3}H]thymidine or with [^{14}C]valine with or without 0.5 mM cytosine arabinoside (Ara-C) (Sigma).

RESULTS

Depot-specific DNA synthesis in vivo

In the first experiment, rates of DNA synthesis in Epi and RP fat depots were compared in rats with continuous ad libitum access to a fattening diet. Measures were made at several time points before significant differences in adipocyte hyperplasia normally appear.

Table 1 shows body weight and adipose tissue cellularity of rats fed HFS or chow. The animals showed a small (not statistically significant) drop in body weight and fat cell size relative to controls on day 5, but the level of DNA synthesis measured at the time of injection (day 3) may reflect an initial increase in body weight in response to HFS. In the rats fed HFS for 10 days, fat cell size was increased by 29% in the Epi pad and 30% in the RP depot (ANOVA, $F = 5.47$, 1/20 df, $P < 0.05$). HFS-feeding for 5 weeks caused fat depot weight and fat cell size to increase significantly in both depots. Fat cell number was significantly increased only in the RP depots (RP: $P < 0.01$; Epi: $P = 0.056$).

Since measures of DNA synthesis can be influenced by depot morphology, we calculated the level of

TABLE 1. Body weights and adipose tissue cellularity of rats fed chow or HFS

	Body weight	Change in Body Wt. before Injection	Epi Depot			RP Depot		
			Pad Weight	Cell Size	Cell Number	Pad Weight	Cell Size	Cell Number
	g	*g*	*g*	*μg lipid/cell*	$\times 10^{-6}$	*g*	*μg lipid/cell*	$\times 10^{-6}$
Chow (N = 5)	499 ± 14[a]	13 ± 2.5	5.94 ± 0.78	0.427 ± 0.049	12.02 ± 0.536	7.62 ± 1.0	0.612 ± 0.086	10.98 ± 0.670
5-Day HFS (N = 6)	478 ± 23	24 ± 4.2*	4.84 ± 0.36	0.362 ± 0.039	12.39 ± 0.521	7.70 ± 0.98	0.583 ± 0.058	11.45 ± 0.158
10-Day HFS (N = 6)	540 ± 14	59 ± 2.9**	7.23 ± 0.48	0.549 ± 0.044*	12.17 ± 0.77	11.24 ± 0.68**	0.798 ± 0.076	12.49 ± 0.951
Chow (N = 5)	651 ± 39	47 ± 5.1	6.60 ± 0.86	0.475 ± 0.049	12.52 ± 0.89	11.0 ± 1.6	0.764 ± 0.100	12.85 ± 0.77
5-Week HFS (N = 6)	780 ± 48*	111 ± 2.5**	14.40 ± 1.6**	0.759 ± 0.069**	18.14 ± 2.90	29.9 ± 4.0**	1.31 ± 0.168**	20.95 ± 1.92**
% Increase[b]	20%	136%	118%	60%	45%	172%	71%	63%

In comparison with appropriate chow-fed controls: *, $P < 0.05$; **, $P < 0.01$ (one-tailed Student's *t* test).
[a] Value represents mean ± SEM.
[b] 5-Week HFS vs chow.

[^{3}H]thymidine in DNA three ways: as incorporated dpm per mg of tissue (dpm/mg), as total dpm incorporated into a fat depot (dpm/pad), and as total dpm/pad divided by fat cell number (dpm/cell). Dpm/mg of tissue is the most direct measure, but it understates the rate of DNA synthesis in fat depots with large fat cells relative to depots with small fat cells. Dpm/pad is an accurate measure of total level of adipose tissue DNA synthesis, but it understates the rate of synthesis in depots with large numbers of cells relative to depots with small numbers of cells. The measure dpm/fat cell varies appropriately as a function of the size of the pool of cells available to synthesize DNA, but only as long as the ratio of SV cells to fat cells does not change.

Table 2 shows that levels of incorporation of radiolabeled DNA precursor are substantially increased in both Epi and RP depots of the 10-day HFS-fed rats. Label incorporation was increased 392% in the Epi pad and 363% in the RP depot (expressed as dpm/pad), even though there was no measurable increase in fat cell number. While the increases in label incorporation in the 5-day HFS group were not as great, they nevertheless suggest that there may have been large increases in DNA synthesis as early as the time of injection, 3 days after the beginning of HFS feeding. There is no indication that the increase in DNA synthesis is greater in RP than in Epi depots at either of these early stages of HFS feeding.

A dramatically different result was obtained in rats fed HFS for 5 weeks and injected with labeled DNA precursor during the final week of that period. These rats showed small increases in fat cell number in both Epi and RP depots, but levels of DNA synthesis in the two depots were dramatically different (Table 2). The mean level of DNA synthesis in Epi pads was not significantly different from that seen in Epi pads of controls, while DNA synthesis in the RP depot exceeded the respective level in controls by 200% (dpm/cell, $P < 0.05$, Student's *t* test, see **Fig. 1**). It is well known that after periods of HFS feeding of 9 weeks or more, increases in fat cell number are dramatically greater in the RP depot than in the Epi (2, 9, 15). Thus, the different levels of DNA synthesis seen here between Epi and RP depots are predictive of their impending differences in adipocyte hyperplasia.

Radioautographic confirmation of de novo production of adipocytes

As discussed above, the concordance of DNA synthetic rates of the various adipose depots with eventual increases in fat cell number suggests, but still does not prove, that newly apparent adipocytes in HFS-fed rats are the result of proliferation and differentiation of precursors rather than the result of differentiation of precursors formed earlier in life. Attempts to distinguish between these two

TABLE 2. In vivo DNA synthesis in adipose tissue of rats fed chow or HFS

	dpm/mg Tissue	dpm/Fat Cell	dpm/Pad
Chow (N = 5)			
Epi	37.6 ± 4.32[a]	19.6 ± 2.6	116.9 ± 16.7
RP	12.22 ± 2.33	9.4 ± 1.76	50.35 ± 11.8
5-Day HFC (N = 6)			
Epi	118 ± 36*	42.9 ± 13.1^{NS}	280.6 ± 89.3^{NS}
Rp	27.1 ± 5.8*	19.5 ± 5.4^{NS}	112.9 ± 33.7^{NS}
10-Day HFC (N = 6)			
Epi	163 ± 20.8***	95.4 ± 8.0***	575.2 ± 52.7***
RP	40.5 ± 7.3**	39.1 ± 8.6**	233.3 ± 49.7**
Chow (N = 5)			
Epi[b]	25.7 ± 7.5	13.2 ± 3.5	158 ± 32
RP	12.24 ± 0.56	10.37 ± 1.29	135 ± 22
5-Week HFC (N = 6)			
Epi	18.6 ± 3.0^{NS}	16.4 ± 3.75^{NS}	272 ± 61^{NS}
RP	21.4 ± 3.75*	31.9 ± 9.06*	676 ± 199*

In comparison with appropriate controls, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (one-tailed Student's t test). NS, not significant.

[a] The greater absolute levels of [^{3}H]thymidine incorporation seen in most EPI depots may be due to the proximity of the EPI tissue to the IP injection site. Value represents mean ± SEM.

[b] N = 4.

possibilities by studies of DNA incorporation in separated SV and fat cell fractions of adipose tissue have generally been confounded by persistant contamination of the fat cell fraction with non-lipid-filled SV cells (8, 16). Radioautographic analysis circumvents this problem. To date, such analysis has been used to demonstrate de novo production of adipocytes in rapidly growing young rats (17), but not in the adipose tissue of adult animals.

In the first experiment, adult OM rats were given ad lib access to HFS. Levels of DNA synthesis were determined for three rats injected with radiolabel during the first month of HFS-feeding, two rats injected during the second month of HFS-feeding, and two controls. DNA synthesis in RP adipose depots (determined biochemically) was increased over control levels by roughly 1000% in both groups of HFS-fed rats. RP adipose tissue from the rat in each group with the highest level of measured DNA synthesis was processed for radioautography.

Between 1700 and 2500 nuclei from the RP adipose

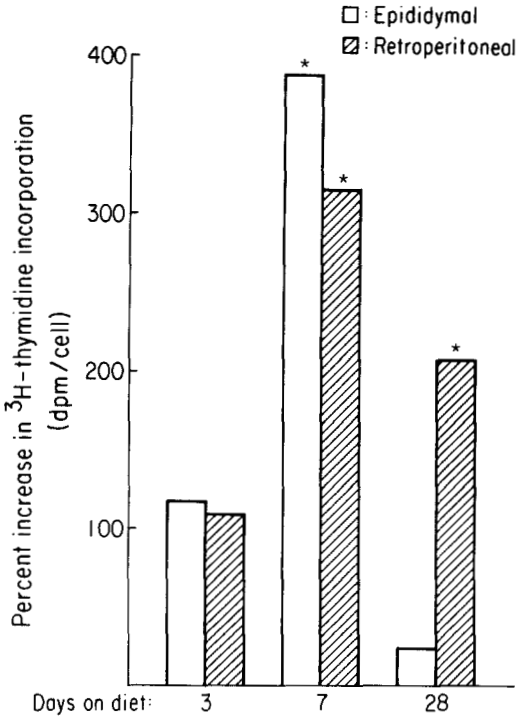


Fig. 1. Effects of HFS-feeding on in vivo DNA synthesis in Epi and RP adipose depots. Mean levels of [^{3}H]thymidine incorporation in depots of rats fed HFS for varying periods of time (from Table 2) are presented as percentage increases over mean levels of incorporation in chow-fed controls. Days on HFS prior to [^{3}H]thymidine injection are indicated. *Represents increases that are statistically significant.

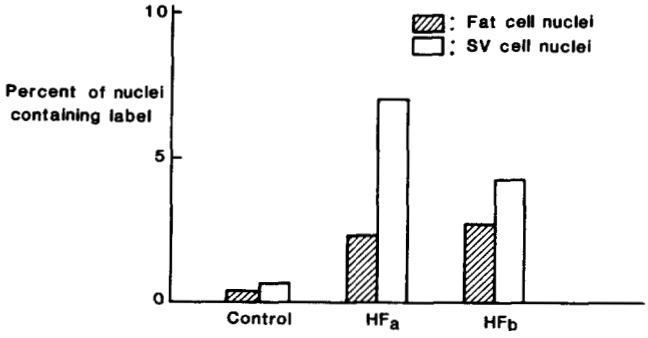


Fig. 2. Analysis of radioautographs of RP adipose tissue from HFS-fed rats (experiment 1). HF_a, rat injected with [^{3}H]thymidine during first 3 weeks of HFS-feeding; HF_b, rat injected with [^{3}H]thymidine during second month of HFS-feeding.

TABLE 3. Cell types observed in radioautographs of adipose tissue of HFS-fed rats

	Fat Cells		Stromal-Vascular Cells	
	Unlabeled	Labeled	Unlabeled	Labeled
RP Depot				
Control	1221	0	8481	11
HFS-fed: first week	1356	27	15121	504
HFS-fed: fifth week	751	9	13693	175
Epi Depot				
Control	898	0	5319	16
HFS-fed: first week	1669	0	10357	40
HFS-fed: fifth week	1374	19	10170	161

depots of each rat were examined for cell type and for the presence of radiolabel. **Fig. 2** shows the percentage of labeled nuclei of each cell type in the adipose tissue samples from the three rats. In both the rat injected during the first month and the rat injected during the second month of HFS-feeding, substantially more nuclei of both fat cells and SV cells were labeled than in the control rat ($P < 0.05$, X^2-test, see Fig. 2).

In the second experiment, DNA synthesis was measured and radioautographs of Epi and RP adipose depots of two control and four HFS-fed rats were analyzed. The HFS-fed rats had been injected with [^{3}H]thymidine during the first or fifth week of HFS-feeding. Levels of DNA synthesis per fat pad increased by 293% and 513% in the Epi pads and 1273% and 1280% in the RP depots of the rats injected in the first and fifth weeks of HFS feeding, respectively. **Table 3** presents the total number of unlabeled and labeled nuclei of SV and fat cells for the various tissue samples. All statistical comparisons in this experiment were also made by means of the X^2-test. In RP depots, labeled nuclei were found in only a few of the SV cells and in none of the fat cells of control rats, while there were many labeled fat cells and SV cells in depots of rats injected during the first week of HFS-feeding. For both cell types, the increases, relative to control values, are highly significant ($P < 0.001$). In RP depots of rats injected during the fifth week of HFS-feeding, labeled nuclei were again seen in SV cells and in fat cells ($P < 0.001$ vs. controls, both cell types), but there were fewer labeled cells than in depots of HFS-fed rats injected during the first week. These effects are summarized in **Fig. 3.** The difference in percent labeled SV cell nuclei between the two HFS-fed groups is highly significant ($P < 0.001$), but the difference between the two groups in percent labeled fat cell nuclei is not significant ($P > 0.20$); radioautographs of the samples from

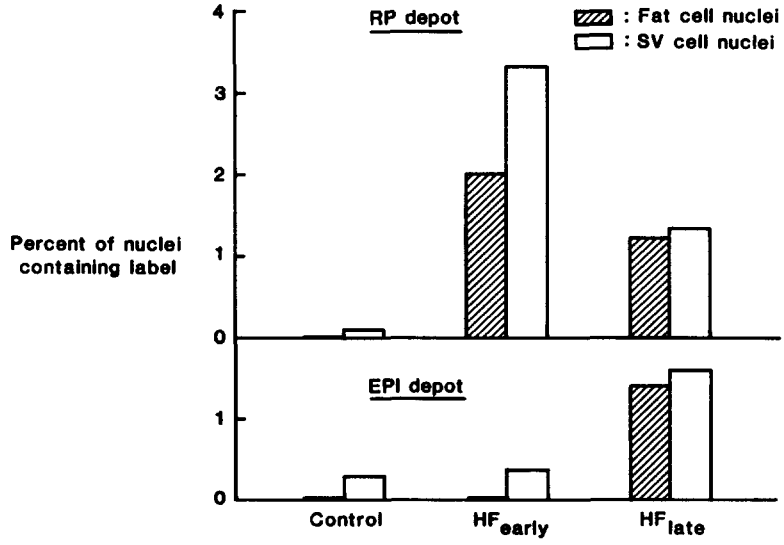


Fig. 3. Analysis of radioautographs of RP and Epi adipose tissue from HFS-fed rats (experiment 2). HF_{early}, rats injected with [^{3}H]thymidine during first week of HFS-feeding; HF_{late}, rats injected with [^{3}H]thymidine during fifth week of HFS-feeding.

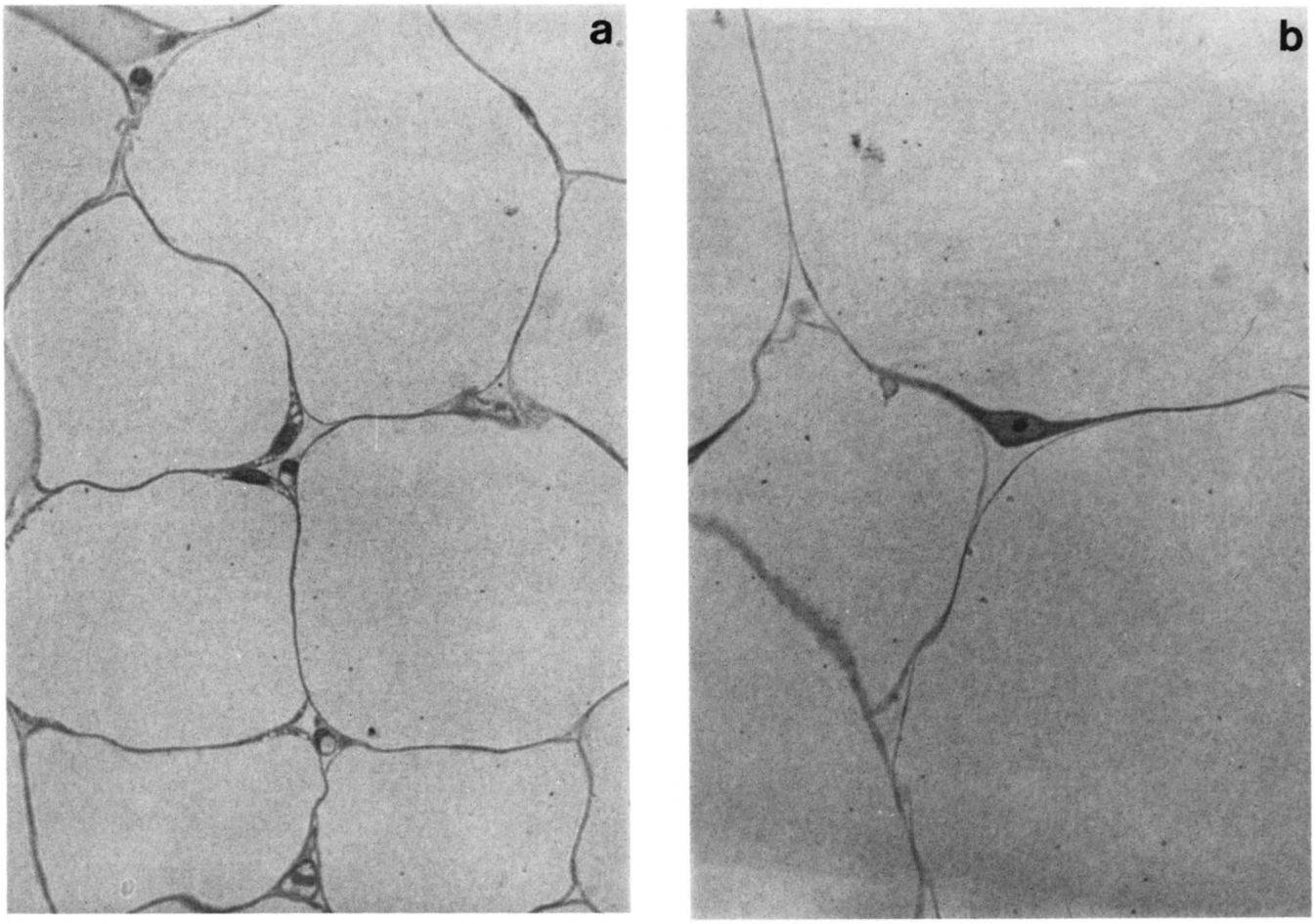


Fig. 4. Representative radioautographs: a, unlabeled fat and SV cells from rat fed chow (100×); b, unlabeled fat cell from RP depot of rat fed HFS (400×); c, labeled fat cell from RP depot of rat fed HFS (400×); d, labeled fat cell from Epi depot of rat fed HFS (400×).

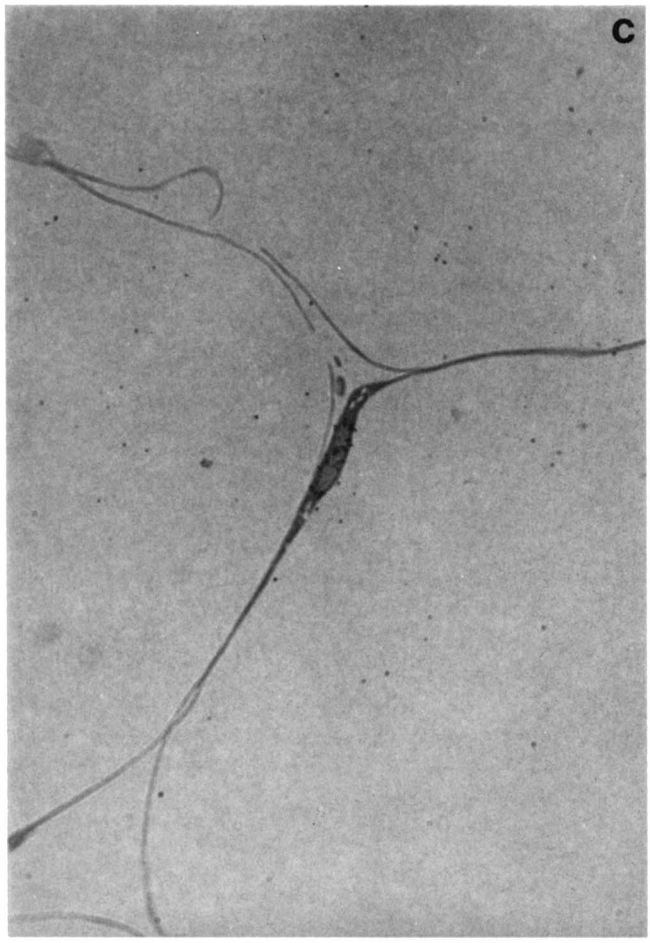
the RP depots of each of the four HFS-fed rats showed comparable numbers of labeled fat cells. The increase in the number of labeled RP fat cells relative to controls was highly significant for each of the four HFS-fed rats ($P < 0.001$ for each rat vs. pooled controls).

As in RP depots, labeled nuclei were found in very few of the SV cells and in none of the fat cells in Epi pads of control rats. However, unlike the case for the RP depot, very few labeled nuclei were found in SV cells and none were found in fat cells from Epi pads of rats injected during the first week of HFS-feeding. In rats injected during the fifth week of HFS-feeding, significant numbers of labeled nuclei were seen in both SV cells and in fat cells ($P < 0.001$ vs. controls and vs. HFS-fed rats injected during week 1). (However, it is important to note that 18 of the 19 observed labeled fat cells were from one rat.)

Representative radioautographs from Epi and RP depots of HFS-fed and chow-fed adult rats are shown in **Fig. 4.**

In vitro DNA synthesis

The above experiments demonstrate that increases in de novo production of cells occur in adipose tissue of rats with diet-induced weight gains and that some of the newly produced cells are fat cells. They also suggest that the total level of DNA synthesis in adipose tissue may be a useful indicator of the rate of proliferation of adipocyte precursors, especially if the measures are made when the rate of proliferation of SV cells is not too high. Since these in vivo measures cannot be directly applied to the study of adipose tissue growth in humans, we developed an in vitro assay of adipose tissue DNA synthesis that can be used with pieces of tissue no larger than those readily obtained by needle aspiration. This assay, which measures the incorporation of [^{3}H]thymidine into adipose tissue fragments, was used to determine whether the diet-induced increases in DNA synthesis seen in vivo can also be seen in isolated pieces of adipose tissue in vitro. DNA synthesis in tissue samples from HFS-fed rats was com-



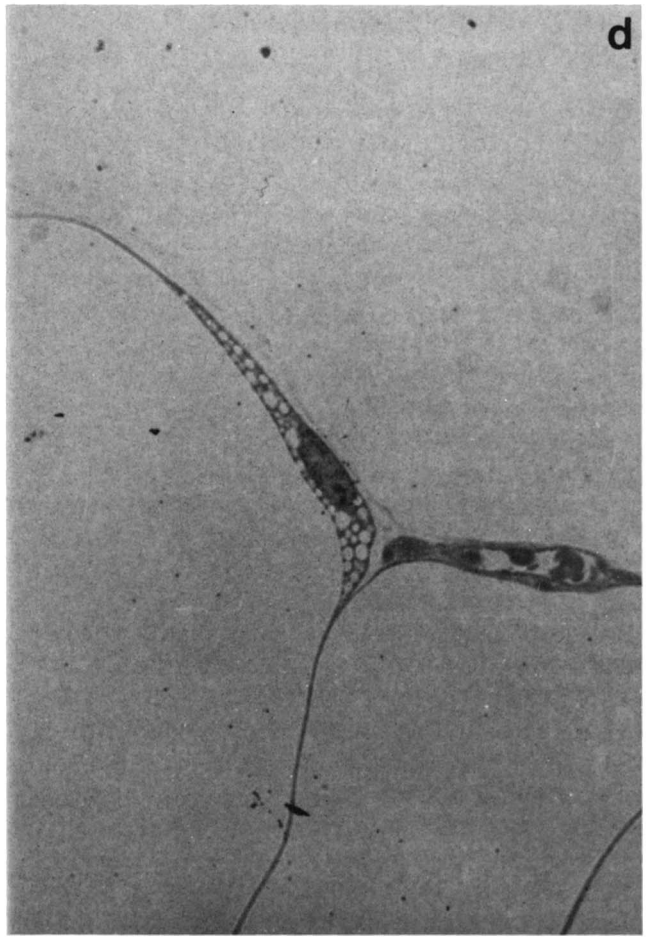


Fig. 4. (*Continued*)

pared with DNA synthesis in tissue samples from rats fed chow. Relative increases in DNA synthesis in Epi and RP depots were also compared.

Control experiments verified that the label incorporated in vitro was in DNA. Duplicate samples of tissue from Epi and RP depots from an adult OM rat were incubated in 1.0 μCi/ml of [^{3}H]thymidine in KRB with and without 0.5 mM Ara-C, a DNA synthesis inhibitor. As shown in **Fig. 5,** the addition of Ara-C to the medium resulted in the inhibition of 95% of the radiolabel incorporation into Epi samples (826 cpm/mg control vs. 41 cpm/mg with Ara-C) and 93% of the incorporation into RP samples (382 cpm/mg control vs. 26 cpm/mg with Ara-C); 0.5 mM Ara-C did not suppress incorporation into protein of 0.2 μCi/ml of [^{14}C]valine label (11.4 cpm/mg control vs. 12.0 cpm/mg with Ara-C). In another control experiment, 100–200-mg pieces of adipose tissue from four different rats (of approximately equal fat cell size) were incubated in vitro as above, while larger amounts (5–10 g) of tissue from the same rats were also incubated with [^{3}H]thymidine in vitro and then processed for DNA purification and specific activity determinations as previously described (8). The correlation of the values of cpm/mg of the small pieces of tissue and cpm/μg DNA of the large pieces was excellent ($r = 0.99$, $t = 36.8$, $P < 0.001$), again confirming that the former technique assays primarily incorporation into DNA.

In vitro incubations were performed to measure DNA synthesis in adipose tissue samples from male OM rats fed either chow or HFS for varying periods of time. Tissue samples showed considerable variation in the amount of label incorporated. Label incorporation (cpm/mg of tissue) was measured in samples from HFS-fed rats and their controls in each incubation and the percentage difference was calculated. As discussed above, while the measure dpm (or cpm)/mg may understate increases in DNA synthesis when fat cells are enlarged (such as in HFS-fed rats), it is the only practical measure for the study of adipose tissue in humans, since depot wet weights in humans cannot usually be assessed.

Since measurements of DNA synthesis by the in vitro technique involved experiments with few data points that

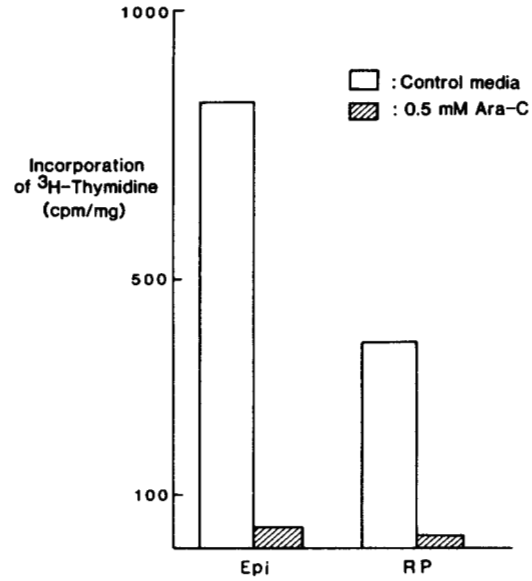


Fig. 5. Inhibition by Ara-C of in vitro incorporation of [^{3}H]thymidine in pieces of adipose tissue from a chow-fed rat. Values are means of duplicate samples of tissue from Epi and RP depots.

were not normally distributed, nonparametric techniques are used to express the data. The median percent differences evident after periods of HFS-feeding of 8 days or less and periods of HFS-feeding greater than or equal to 2 weeks are shown in **Fig. 6.** The incorporation of thymidine into DNA in both depots is substantially increased relative to controls in animals fed HFS for up to 8 days (+104% in the Epi pad and +99% in the RP depot). In animals fed HFS for between 2 weeks and 42 days, the median increase relative to controls in the Epi tissue samples was 0% and in the RP tissue samples was +50%. These changes are consistent with the changes in DNA synthesis seen in the in vivo experiments discussed above.

Because of the large variation in label incorporation in the in vitro incubations, we compared DNA synthesis levels in Epi and RP tissue samples from each rat. The magnitude of incorporation of radiolabel into tissue from one depot of a rat thus serves as a control for incorporation into the other depot. We calculated this Epi/RP ratio for 22 chow-fed rats, 12 rats fed HFS for 3–8 days and 12 rats fed HFS for 14–42 days, and made statistical comparisons using Student's *t* test. The ratio of Epi label uptake to RP label uptake in chow-fed rats was 1.20 ± 0.06. Rats fed HFS for 3–8 days had a ratio of 1.01 ± 0.11 ($P < 0.10$ vs. chow-fed rats) which suggests a greater increase in RP DNA synthesis. With 2 or more weeks of HFS-feeding, 11 of 12 rats showed a mean ratio of 0.898 ± 0.043 ($P < 0.001$ vs. chow-fed rats). (One rat fed HFS for 28 days had an Epi/RP ratio outside the range of all observed values and greater than seven standard deviations above the mean of the others in its group.) The overall average ratio for all rats fed HFS was 0.95 ± 0.06 vs. a ratio of 1.20 ± 0.06 for the chow-fed rats ($P < 0.01$).

DISCUSSION

This report presents experiments that explore the origin of newly apparent cells in adult rats fed a high-fat, high-sugar diet (HFS). In these experiments, rates of DNA synthesis in two depots of rats fed HFS were found to be consistent with known patterns of diet-induced fat cell number increase (2, 10). Examination of radioautographs of adipose tissue from the HFS-fed rats revealed that the increased levels of DNA synthesis induced by the HFS diet reflect synthesis of new cells, including adipocytes. Diet-induced changes in DNA synthesis were also observed with an in vitro assay, which is therefore potentially of value for the assessment of rates of adipose tissue DNA synthesis in humans.

Increases in the number of adipocytes in rats induced to gain weight by HFS are depot-specific. In previous experiments, the RP depot has shown up to 10-fold increases in adipocyte number in response to HFS, while the Epi depot has shown increases that were more moderate and less consistent (2, 10, 15). This report shows that differences in DNA synthesis between these two depots correspond to their different susceptibilities to diet-induced hyperplasia.

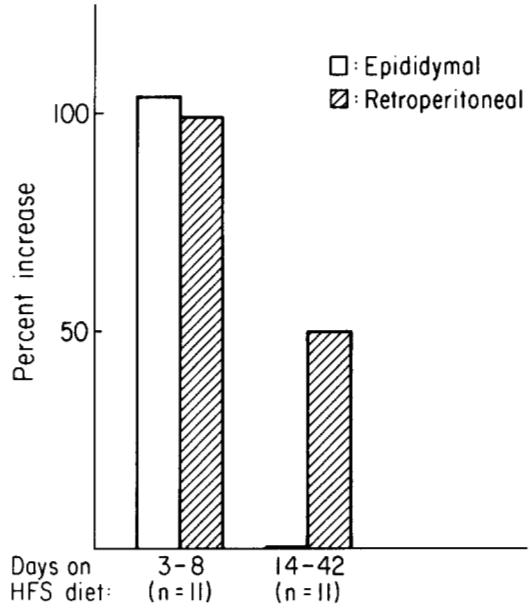


Fig. 6. Adipose tissue DNA synthesis in vitro. [^{3}H]Thymidine incorporation in adipose tissue of HFS-fed rats is compared to that of chow-fed rats in several experiments. Figure shows percent increase over control values for two depots.

Since essentially identical increases in in vivo DNA synthesis are typically seen in SV and adipocyte fractions prepared from adipose tissue of adult rats fed a high-fat diet (9), the in vitro assay tested in the present study used pieces of intact adipose tissue only. Using intact tissue instead of isolated fractions considerably reduced the time needed to measure incorporation of label into DNA and precluded possible artifactual effects due to selective digestion or destruction of cells by collagenase (18, 19). The pieces of tissue used were of a size that is easily obtainable from human subjects by aspiration.

In the in vivo DNA synthesis experiment, both Epi and RP fat cell number were increased in the rats fed HFS for 5 weeks relative to chow-fed controls, although only the increase in the RP depot attains statistical significance. Based on previous studies, the small difference in fat cell number increase between Epi and RP depots would have expanded dramatically with extended HFS-feeding (2, 10). Thus, the different DNA synthetic rates seen in the two depots after 4 weeks of HFS feeding accurately predict impending differences in cellularity.

Although they may have important predictive value, studies of DNA synthesis in whole adipose tissue do not clearly distinguish production of adipocytes from production of other cell types in the tissue. This is also true for studies of "isolated" adipocytes, especially those taken from adult rats, since such adipocyte preparations tend to be highly contaminated by SV cells (8, 15). Radioautographic studies are much better suited to distinguish production of adipocytes from production of other cell types. In this study, radioautographs clearly showed that the increase in adipose tissue DNA synthesis induced by dietary obesity does not reflect solely the synthesis of new non-lipid-filled support cells. Significant numbers of fat cells with newly synthesized nuclear DNA were present in the RP depots of HFS-fed rats in two separate experiments. In both experiments, the percentage of labeled RP fat cells in the HFS-fed rats was significantly greater than that of controls. Moreover, in the second experiment, the number of labeled RP fat cells was significantly elevated in each of the four HFS-fed rats. Thus, rats gaining weight on HFS reliably show increased de novo production of adipocytes in the RP depot.

The results of the radioautographic experiments also reveal that pre-adipocyte production in the RP depot is accelerated very early during the course of HFS-feeding. In the second radioautographic experiment, substantial numbers of labeled fat cells appeared in the RP adipose tissue of both rats injected with [^{3}H]thymidine during the first week of HFS-feeding. The percentage of observed radiolabeled SV cell nuclei is also consistent with the results of the whole tissue DNA synthesis studies. Many more labeled SV cells were observed in sections of adipose tissue from rats injected during the first week of HFS-feeding than from rats injected during the fifth week. These findings support the notion, discussed above, that high levels of SV cell production during the initial stage of HFS-feeding prevent the detection of pre-adipocyte proliferation by biochemical techniques.

Taken together, the results of the present study suggest that the cellular proliferation in a fat depot stimulated to hypertrophic and hyperplastic growth by HFS-feeding has two phases. In the initial stage, there is substantial adipocyte hypertrophy, accompanied by high levels of DNA synthesis in non-lipid-filled cells. Fat cell size doubles during the first 3 weeks of HFS-feeding, while fat cell number is unchanged (2). The observed increase in level of DNA synthesis reflects the production of increased numbers of SV cells, needed to provide support for the increasing mass of tissue, and the production of a number of adipocyte precursors which varies from depot to depot. In the second stage, production of adipocyte precursors continues, and the rate of production of SV cells declines. During this stage, newly formed pre-adipocytes fill with lipid and the number of observable fat cells increases.

A more variable pattern of growth appears to occur in Epi pads than in RP depots. Whole tissue DNA synthesis in vivo and in vitro is usually, but not always, very high in Epi pads of rats injected with radiolabel during the first week of HFS-feeding, while it is relatively low in rats injected after several weeks of HFS-feeding. This suggests that substantial increases in the number of supporting SV cells usually occur in the Epi pad during the first few weeks of HFS-feeding and that continued HFS-feeding promotes a relatively low level of fat cell production. Evidence of fat cell production was not seen at all in radioautographs of rats injected with radiolabel during the first week of HFS-feeding and was seen in only one of two rats injected during the fourth week of HFS-feeding. Thus, while the RP depot shows accelerated production of fat cells almost immediately upon the initiation of overfeeding, with production of large numbers of fat cells continuing for many weeks, the Epi pad does not. Fat cell production in the Epi pad is initiated much more slowly and less predictably. This finding is consistent with the small and variable degree of adipocyte number increase typically seen in Epi depots of rats fed HFS (2, 10, 15). In cattle, newly formed adipocytes appear primarily during discrete periods separated by long intervals during which no new fat cells appear (20). A similar pattern of fat cell production in the Epi pad would account for the variable results obtained for Epi pads in previous cell-counting studies and in this report.

The rate of new fat cell production seen in the RP depot in this study can be compared with that predicted by cell-counting data. As discussed above, a doubling of fat cell number in the RP depots of male OM rats was seen to occur by about the ninth week of weight gain

induced by a HFS diet (2). If we assume that pre-adipocyte synthesis occurs at a roughly even rate during the first 9 weeks of HFS-feeding and that the DNA synthetic (S) phase lasts 6 hr in the pre-adipocyte, $6/(24 \times 7 \times 9) = 0.4\%$ of fat cell precursors should be synthesizing DNA at any given time during those 9 weeks. Since [^{3}H]thymidine injected into rats is rapidly metabolized, about 0.4% labeled fat cells should be seen in radioautographs of adipose tissue from a rat injected only once during the first 9 weeks of HFS-feeding. Based on these assumptions, the first radioautographic experiment (in which rats were injected up to twelve times) should have yielded about 4–5% labeled fat cells. The second radioautographic experiment, in which rats were injected twice, should have yielded approximately 1% labeled fat cells. The observations were certainly well within the range of these predictions, although there was a somewhat higher percentage of labeled fat cells than predicted in the second experiment. This slightly higher value has several possible explanations: the rats might have doubled fat cell number in less than 9 weeks or the rate of pre-adipocyte proliferation may have been uneven over the 9-week period of HFS-feeding. In addition, great variability in the number of labeled cells commonly occurs in radioautographic preparations (13), and errors in counting labeled fat cell nuclei in radioautographs are always possible. (One possible error, the preferential examination of areas on a slide where labeled nuclei occur, was avoided by counting all recognizable nuclei on a given slide.)

Since the observed labeling of cells from adipose tissue of HFS-fed rats can account fairly well for the known increases in fat cell number that occur in these animals, there is no need to postulate the existence of a large pool of nondifferentiated pre-adipocytes in the adult.

Since the results of the in vitro experiment are consistent with the in vivo studies, they provide a method for assessing cellular growth without the in vivo injection of radiolabeled precursors. As in the in vivo experiments, HFS-feeding was found to induce large increases in adipose tissue DNA synthesis that are maximal in rats fed the diet for up to 1 week and vary substantially between Epi and RP depots in rats fed HFS for 2 weeks or more.

The good agreement with the in vivo experiments, along with the observation that biopsied adipose tissue fragments from obese and lean patients have different proliferative capacities in tissue culture (11, 21, 22), suggest that in vitro assessment of adipose tissue growth in human obesity may be possible. Studies of human obesity suggest that fat cell size is increased before and along with increases in fat cell number (3, 23, 24), and obese adults often experience long periods of enlarged fat cell size (3). As in HFS-fed rats, such individuals may have high rates of production of SV cells that interfere with the measurement of pre-adipocyte proliferation only during the early stages of weight gain. If so, an in vitro assay of adipose tissue DNA synthesis may reflect pre-adipocyte proliferation during all but the earliest stages of the development of obesity and may thus prove to be useful for monitoring the extent and location of ongoing adipocyte hyperplasia. With the establishment of optimal conditions for measuring DNA synthesis in an in vitro system such as presently described, it may be possible to obtain reliable data from human adipose tissue that has potentially useful clinical application.

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